AD_____

GRANT NUMBER DAMD17-97-1-7093

TITLE: Melvalonates, Ras and Breast Cancer

PRINCIPAL INVESTIGATOR: Jennifer A. Cuthbert, M.D.

CONTRACTING ORGANIZATION: University of Southwestern Texas
Medical Center
Dallas, Texas 75235-9016

REPORT DATE: September 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1998	Annual (1 Sep 97 - 31 Aug 98)

4. TITLE AND SUBTITLE

Melvalonates, Ras and Breast Cancer

5. FUNDING NUMBERS

DAMD17-97-1-7093

6. AUTHOR(S)

Cuthbert, Jennifer A., M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Southwestern Texas Medical Center
Dallas, Texas 75235-9016

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

This report documents progress on studies of the effects on breast cancer cell proliferation of reagents that inhibit mevalonate synthesis (lovastatin) or metabolism (6-fluoromevalonate (Fmev), a mevalonate analogue). In our initial year we have established methods to quantitate cell proliferation and applied these in studies of the effects of the inhibitors. Lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase activity, induced apoptosis and suppressed the proliferation of breast cancer cell lines in a concentration-dependent and specific manner. Mevalonate restored cell growth and prevented apoptosis. In contrast, neither cholesterol nor precursors of farnesyl and geranylgeranyl pyrophosphate, was able to restore cell growth. These results implicate an essential requirement for mevalonate over and above its necessity for membrane synthesis (cholesterol). Furthermore, they suggest that bypassing the block in post-translational prenylation of proteins is not sufficient to permit cellular proliferation. Fmev also inhibited breast cancer cell lines. In MCF-7 cells, the mechanism of suppression was the accumulation of a mevalonate-derived inhibitor. This effect was produced by elevated mevalonate synthesis, the result of poorly regulated HMG CoA redcutase activity. Ongoing studies are determining the effects of these inhibitors on intra-cellular Ras levels and its distribution.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer Ras, mevalonate phosphates, fluoromevalonate, lovastatin, proliferation	19
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18 298-102

USAPPC V1.00

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

__✓__ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

__✓__ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Jennifer Woodbell 9/29/98
PI - Signature Date

TABLE OF CONTENTS

PROGRESS REPORT - September 1998
MEVALONATES, RAS AND BREAST CANCER

INTRODUCTION to Progress Report

Subject of proposed research: Therapy of breast cancer – Therapy of any cancer is designed to control the growth of malignant cells, while permitting proliferation and function of normal cells. We are evaluating a novel approach for breast cancer therapy, using an analogue of mevalonate, 6-fluoromevalonate (Fmev). We have demonstrated that Fmev may target cancer cells specifically by at least two different mechanisms.[1-3] One mechanism is dependent on the unregulated production of mevalonate, a marker of malignancy in some cancer cells.[1,3] The second mechanism is linked to the role of oncogenic Ras in the proliferation of cancer cells.[2]

Purpose of proposed research: The general purpose of the proposed research is to evaluate Fmev as a potential selective inhibitor of the proliferation of breast cancer cell lines *in vitro*. In addition, the mechanism whereby Fmev inhibits breast cancer cell proliferation will be determined. By identifying specific targets in the growth of breast cancer cells *in vitro*, treatments with greater selectivity or potency may then be developed based on the information gained from the initial studies. The long-term purpose is to permit rational development of therapies that can then be used *in vivo* for the treatment of patients with breast cancer requiring chemotherapy. In addition, if targets that distinguish benign from malignant cells are identified, this knowledge may be useful in early detection of malignant potential. Furthermore, products that are developed based on the ability of Fmev to target cancer cells specifically may be useful in chemoprevention.

Background of previous work: Fmev is an analogue of the naturally-occurring substance mevalonate.[4] Mevalonate is a key intermediate in the synthesis of cholesterol and all sterol molecules.[5] Mevalonate is also the precursor for the synthesis of lipid moieties that post-translationally modify proteins by the process termed prenylation.[5] In this process, either a 15-carbon lipid from farnesyl pyrophosphate (farnesylation) or a 20-carbon lipd from geranylgeranyl pyrophosphate (geranylation) is covalently linked to the carboxyl terminus of proteins bearing a recognition sequence for prenylation (Figure 1, Appendix). Post-translational modification by prenylation is essential for the correct membrane localization of proteins. In normal cells, mevalonate synthesis is tightly regulated by controlling the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, the enzyme that catalyzes the synthesis of mevalonate from HMG CoA.[5] HMG CoA reductase activity is regulated by transcriptional, translational and post-translational mechanisms in normal cells.[5] In cancer cells, synthesis of mevalonate can be poorly regulated. Thus, the end-product cholesterol decreases transcription of HMG CoA reductase and is also necessary for regulated degradation of the enzyme in normal cells. In cancer cells, however, cholesterol can lose its regulatory capacity, with consequent increases in HMG CoA reductase activity and mevalonate synthesis.[1,3]

HMG CoA reductase activity can be suppressed in normal cells proliferating *in vitro* by the addition of specific inhibitors, such as lovastatin.[6,7] When low concentrations of lovastatin are used to inhibit activity, continued cell growth becomes dependent on the ability to obtain cholesterol from exogenous sources (lipoproteins in serum).[2,8] At higher concentrations of lovastatin, however,

cholesterol alone is not sufficient to restore normal proliferation.[6,7] Another product, derived from mevalonate is also required.[6,7] Thus, the combination of both exogenously-supplied cholesterol and mevalonate is necessary to restore cell growth. The concentrations of mevalonate that are active are not sufficient, in the absence of cholesterol, to support proliferation.[7] The identity of this essential mevalonate-derived non-sterol product has remained elusive since its existence was first demonstrated more than 20 years ago.[6]

Fmev, like lovastatin, suppresses the generation of most mevalonate-derived products.[7,9,10] Thus, Fmev inhibits the conversion of mevalonate pyrophosphate to isopentenyl pyrophosphate 9see Figure 1, Appendix). Consequently, the de novo synthesis of cholesterol, related prenyl-derived lipids and the farnesyl and geranylgeranyl moieties used in post-translational modification of proteins are all prevented in Fmev-blocked cells.[2,3,7] We have therefore explored the potential of Fmev to block cellular proliferation.[1-3,7] Fmev effectively prevented the *in vitro* proliferation of normal human T lymphocytes[7] and diploid fibroblasts (unpublished data). In contrast to lovastatin blockade, however, proliferation of Fmev-blocked normal cells was restored by the provision of exogenous cholesterol alone.[7] This observation clearly indicated that there were fundamental differences in the effects of lovastatin and Fmev on mevalonate metabolic pathways.[7]

We have also demonstrated that many leukemia- and lymphoma-derived cancer cells behave differently to normal cells.[1,3] Thus, the growth of these Fmev-blocked cancer cells was not restored by exogenous cholesterol.[1,3] Of greatest importance was the observation that Fmev-arrested leukemia and lymphoma cell proliferation was restored by preventing the accumulation of a mevalonate-derived inhibitory product.[1,3] Accordingly, the addition of lovastatin to Fmev-blocked leukemia and lymphoma cells (together with an exogenous source of cholesterol) permitted normal proliferation.[1,3] There was elevated and unregulated activity of HMG CoA reductase in these cells.[3] In contrast, cells with low or normally regulated levels of HMG CoA reductase activity were protected from the inhibitory effects of Fmev. These observations suggest that Fmev may afford inhibition of proliferation that is targeted specifically to cancer cells with unregulated HMG CoA reductase activity.

Other studies carried out in our laboratory have demonstrated that proliferation that is dependent on oncogenic Ras can be specifically suppressed by Fmev.[2] Ras, a small GTP-binding protein functions as a molecular switch in normal cells.[11,12] Mutations that lead to constitutive activation of Ras are oncogenic and can be demonstrated in many cancers, including some breast cancers.[12] In our experiments, cell proliferation that was dependent on exogenously supplied growth factor (interleukin-3) became growth factor-independent when cells were transfected with oncogenic Ras.[2] Ras-transfected cells acquired increased sensitivity to the inhibitory effects of Fmev.[2] Indeed, restoration of normal proliferation required the provision of interleukin-3.[2] These observations suggest that aberrant cellular proliferation, a hallmark of malignancy, may be susceptible to Fmev blockade when oncogenic mutation of Ras is involved in the development of uncontrolled growth.

Scope of proposed research: The proposed work will determine whether Fmev or related reagents may be useful as a chemotherapeutic intervention in breast cancer. In addition, the mechanism of targeted suppression of the proliferation of breast cancer cells will be investigated.

BODY of PROGRESS REPORT

Experimental methods: The following methods have been used in the studies to date –

1. Culture of breast cancer cell lines *in vitro* and quantitation of proliferation.
2. Determination of the effect of lovastatin on breast cancer cell proliferation *in vitro*.
3. Investigation of the mechanism of lovastatin-mediated inhibition by characterization of the capacity of various mevalonate-derived products to restore lovastatin-blocked proliferation to normal.
4. Determination of the effect of Fmev on breast cancer cell proliferation *in vitro*.
5. Investigation of the mechanism of Fmev-mediated inhibition by characterization of the capacity of lovastatin to restore Fmev-blocked proliferation to normal.
6. Evaluation of the broad applicability of the results of lovastatin and Fmev blockade by determining whether there are similarities in the responses of diverse breast cancer cell lines.
7. Detection of Ras in breast cancer cell lines by immunoblot and determination of the major family member (Harvey (H)-Ras, Kirsten (K)-Ras or N-Ras) using type-specific antibodies.
8. Optimization of procedures to immunoprecipitate endogenous Ras for experiments determining the effects of Fmev and lovastatin on the synthesis and degradation of Ras.

Assumptions: The major assumption on which the proposal was based, was that the effects of Fmev and lovastatin on the proliferation of breast cancer cell lines would be similar to the effects on leukemia- and lymphoma-derived cell lines. Alternatively, a novel effect of these reagents on breast cancer cell proliferation might be encountered. The other possibility was that neither compound suppressed breast cancer cell growth. Since there were little available data regarding lovastatin and breast cancer cell lines and no data with respect to the effects of Fmev on the proliferation of breast cancer cell lines, these assumptions were of great importance. As detailed below, breast cancer cell lines resemble leukemia and lymphoma cell lines and exhibit a predictable variability in the effects of Fmev and lovastatin on growth.

Procedures: The following specific procedures have been used to date -

i. Quantitation of the proliferation rate of breast cancer cell lines cultured *in vitro*. This was an essential initial series of experiments for a number of reasons. First, we needed to establish the exact procedures that permitted the maximum number of population doublings without sacrificing the precision of quantitation. Secondly, the precise methodologies previously used for the quantitation of the proliferation of leukemia and lymphoma cell lines were predicated on the growth of these cells as a suspension culture in microtiter wells. Since the breast cancer cell lines were adherent cell cultures, alternative procedures were needed. Finally, we are mainly interested in the effects of lovastatin and Fmev on the dividing cell population. Quiescent cells may be relatively (or absolutely) spared unless there is a direct cytotoxic effect or triggering of apoptosis in a non-dividing cell. Therefore, we needed to establish procedures to maximize cellular division prior to the conclusion of the experiment. We have used parallel measurements of cell protein and cell number to quantitate growth. In addition, we have visually captured the differences in proliferative rates by fixing cells and staining *in situ* with crystal violet.

ii. The mechanism of lovastatin-mediated inhibition of proliferation has been investigated by examining the capacity of the prenyl alcohols farnesol and geranylgeraniol to restore growth

when provided in combination with an exogenous source of cholesterol. The ability of exogenously supplied mevalonate to restore growth has been used to demonstrate the specificity of lovastatin-mediated suppression and for comparison purposes with other purported mevalonate-derived essential products.

iii. The mechanism of Fmev-mediated inhibition of proliferation has been investigated by examining the effects of lovastatin in Fmev-blocked cells. Two separate patterns of response are observed in leukemia and lymphoma cell lines. In cells with elevated HMG CoA reductase activity and thence increased synthesis of mevalonate, lovastatin restores Fmev-mediated inhibition of proliferation by preventing the accumulation of an inhibitory substance produced from mevalonate or its phosphate derivatives. In cell lines with either low levels of HMG CoA reductase activity or normally regulated activity, mevalonate synthesis is insufficient for the inhibitor to accumulate. In these cell lines, consequently, lovastatin fails to restore growth and in fact may increase the inhibitory effects of Fmev, which also tends to be less potent.

iv. Measurement of the rate of HMG CoA reductase activity in breast cancer cells has been used to investigate the differences in the effects of Fmev on breast cancer cell lines. In leukemia and lymphoma cell lines, HMG CoA reductase activity was elevated and further induced by Fmev in cell lines demonstrating proliferation which was readily blocked by low concentrations of Fmev. In contrast, relatively resistant cell lines exhibited low levels of HMG CoA reductase activity that was not further induced by Fmev.

v. Examination of the pathway leading to inhibition of cell proliferation in breast cancer cell lines has been undertaken using fluorescence-activated cell sorter (FACS) analysis. Cell death can be induced by triggering apoptosis in susceptible cell populations. During the process of apoptosis, distinctive changes occur in the nucleus with resultant fragmentation of nuclear DNA. This can be quantitated by FACS analysis using propidium iodide staining of DNA and detection of cells with intact nuclei and DNA content between 2n (G1 phase of the cell cycle) and 4n (prior to mitosis after completion of S phase) and cells where the DNA has fragmented.

vi. The mevalonate metabolic pathway has been investigated in breast cancer cell lines using trace quantities of radiolabeled mevalonate. Following incubation with the labeled mevalonate, cells were separated into lipid, aqueous and protein fractions and the incorporation of mevalonate into each fraction was quantitated. This method permits further analysis of potential differences in mevalonate metabolism between cell lines.

Results: The following results have been obtained to date –

Establishment of cell culture conditions for proliferation was achieved initially. For cell lines that divide more rapidly (e.g. MCF-7, a commonly used breast cancer cell line), seeding cultures with 10,000-20,000 cells per single well in a 24-well plate resulted in ~50% confluence in 5-7 days and complete confluence in 7-9 days. Higher cell numbers were used in initial seeding of breast cancer cell lines with slower division.

Quantitation of cell proliferation was undertaken using measurements of cell number and cell protein. Cell number was quantitated by releasing the cells from the tissue culture plates into a single cell suspension with trypsin and counting the resultant cells using a Coulter® counter. An inverted microscope was used to establish that all cells were released by trypsin. The Coulter® counter oscilloscope and aperture window were monitored to ensure that a single cell suspension was obtained. Cell protein was quantitated using the Bradford method. Finally, cells in identically-treated parallel cultures were fixed and stained with crystal violet. There was excellent agreement between the visual effects (crystal violet staining) and the quantitative measurements and between the two separate quantitative determinations.

Effects of lovastatin on breast cancer cell proliferation was next determined.Since few data exist regarding the ability of lovastatin to inhibit the proliferation of breast cancer cell lines *in vitro*, we carried out a series of experiments. These were essential for developing an understanding of the role of mevalonate and its intermediates in the proliferation of breast cancer cells. The proliferation of breast cancer cell lines was inhibited in a dose-dependent manner by lovastatin. Of note, however, high concentrations of lovastatin were required to inhibit proliferation completely. Thus, we have previously demonstrated that lovastatin totally suppresses the proliferation of most leukemia and lymphoma cell lines when present in concentrations of $\leq 20\ \mu M$. In contrast, $100\ \mu M$ lovastatin was needed for suppression of the proliferation of breast cancer cell lines. The addition of mevalonate (1mM) prevented any inhibition by lovastatin, thereby demonstrating the specificity of the inhibition.

Prenyl moieties and lovastatin-mediated inhibition were investigated. Recent studies have indicated that lovastatin-mediated inhibition of proliferation may be prevented by the provision of an exogenous source of prenyl alcohols. Thus, farnesol and geranylgeraniol can be incubated with cultured cells to provide precursors for the synthesis of farnesyl and geranylgeranyl phosphates. The farnesyl and geranylgeranyl phosphates may then post-translationally modify proteins and permit appropriate intra-cellular localization. The addition of farnesol had no effect on lovastatin-mediated inhibition of the proliferation of breast cancer cell lines. Geranylgeraniol minimally increased the proliferation of the cells. In contrast, mevalonate completely restored cell growth in these experiments.

Fluorescence-activated cell sorter analysis of apoptosis was investigated in lovastatin-blocked cells. Using propidium iodide to identify DNA fragmentation, the effects of lovastatin alone and in combination with exogenously supplied mevalonate was determined. Lovastatin substantially increased the fraction of cells demonstrating DNA fragmentation, an indicator of the triggering of apoptosis. When mevalonate was added concomitantly, normal nuclear DNA integrity was retained.

Effects of Fmev on breast cancer cell proliferation was examined. The proliferation of the breast cancer cell line MCF-7 was suppressed in a concentration-dependent manner by Fmev. The relative potency of Fmev was intermediate between that observed with sensitive and resistant leukemia and lymphoma cell lines. Above $100\ \mu M$ Fmev, there was reproducible inhibition of cell growth. In contrast another breast cancer cell line, MDA-468-T, was relatively resistant to inhibition by Fmev. This variability was encountered in our previous experiments using leukemia and lymphoma cell lines and was explained by differences in HMG CoA reductase activity that permitted prediction of

the inhibitory pathway involved. Two separate lines of experimental evidence were obtained that indicated a similar pattern of responses in breast cancer cell lines.

First, the inhibitory effects of Fmev on breast cancer cell proliferation were modestly augmented by the addition of exogenous mevalonate. This was the predicted effect based on the conclusion that one mechanism whereby Fmev inhibited proliferation was by the accumulation of mevalonate or the mevalonate phosphates. The second observation supporting the role of unregulated mevalonate synthesis and subsequent accumulation of an inhibitor was the effect of the addition of lovastatin to Fmev-blocked cells. Lovastatin completely restored the proliferation of Fmev-inhibited MCF-7 breast cancer cells. In contrast, in MDA-468-T cells, the modest inhibition observed with Fmev was only partially restored by lovastatin.

HMG CoA reductase activity in breast cancer cell lines was then determined. The hypothesis was that the level of activity was substantially higher in MCF-7 cells than in MDA-468-T cells. This hypothesis was generated to explain the increased sensitivity of MCF-7 cells to inhibition by Fmev when compared with MDA-468-T cells. The predicted higher HMG CoA reductase activity would increase mevalonate synthesis and therefore lead to greater accumulation of the mevalonate-derived inhibitor when Fmev was present. The results obtained were consistent with our hypothesis and in agreement with previous work in leukemia and lymphoma cell lines. Thus, the HMG CoA reductase activity in MCF-7 cells was 336 ± 30 pmol/hr/10^6 cells (35 ± 3 pmol/min/mg cell protein) whereas it was 98 ± 5 pmol/hr/10^6 cells (8.5 ± 0.4 pmol/min/mg cell protein).

Mevalonate metabolic pathways in breast cancer cell lines were analyzed using [^{3}H] mevalonate. The majority of labeled mevalonate partitioned with the lipid fraction ($96 \pm 1\%$ in MCF-7 cells, $95 \pm 1\%$ in MDA-468-T cells) with $4 \pm 1\%$ in the aqueous fraction in both cell lines and $0.4 \pm 0.2\%$ and $1.3 \pm 0.1\%$ in the cell protein fraction from MCF-7 and MDA-468-T cell lines, respectively. Of note, the incorporation of labeled mevalonate into the protein fraction was substantially higher in MDA-468-T cells (6.0 ± 0.4 fmols/10^6 cells cultured) than in MCF-7 cells (2.1 ± 0.4 fmols/10^6 cells). In these experiments, the specific activity of the radiolabeled mevalonate was not adjusted by the addition of mass quantities of unlabeled mevalonate.

Detection of Ras in breast cancer cell lines was next undertaken. In the first experiments, we used a *pan*-Ras antibody LA045 for immunoblotting. In breast cancer cell lines, this antibody detected a protein corresponding in migration characteristics to cellular Ras. The abundance of Ras was somewhat greater in MCF-7 cells than in MDA-468-T cells. When cell lysates were centrifuged and separated into pellet (membrane) and supernatant (cytosol) fractions, Ras was only present in the pellet (membrane) fraction, as expected. Incubation of the breast cancer cell lines with lovastatin lead to depletion of Ras from the pellet (membrane) fraction and appearance in the supernatant (cytosol). This observation is consistent with lovastatin-mediated inhibition of the synthesis of farnesyl pyrophosphate, the mevalonate product that post-translationally modifies Ras and is essential for membrane localization. The H-Ras specific antibody R02120 also detected protein in breast cancer cell lines. In contrast, antibodies specific for K-Ras and N-Ras did not detect protein in breast cancer cells.

Ras immunoprecipitation (IP) is being optimized currently. Endogenous Ras in breast cancer cell lines was detectable by immunoblotting techniques. However, the amount of protein detected was

quite low, reflecting only **endogenous** Ras, rather than trasnfected (over-expressed) Ras. Consequently, we need to improve on available immunoprecipitation methods such that the synthesis and degradation of endogenous Ras can be readily detected. Initially, we used anti-HA to IP Ras from leukemic cells previously transfected with HA-tagged H-Ras and detected immunoprecipitated H-Ras with the H-Ras specific monoclonal antibody R02120. We have also immunoprecipitated HA-tagged H-Ras with both R02120 and the rat monoclonal antibody Y13-259, a commonly used antibody for similar studies. Anti-HA was used to detect Ras in the IP. Y13-259 was more effective at imunoprecipitating HA-tagged H-Ras than R02120. Therefore, we are carrying out all current experiemtns for optimization using Y13-259. An additional advantage (compared with R02120) is cost-savings since we have obtained the hybridoma cell lines that secrete Y13-259 and prepared the monoclonal antibdy for our use. In parallel, we are also determining the parameters for permanent transfection of oncogenic Ras into breast cancer cell lines. We have established the concentration of G418 necessary for selection of permanent transfectants expressing the neomycin gene in addition to Ras and are currently comparing lipofection protocols and electroporation (techniques with which we have considerable expertise) for introduction of the Ras constructs. We have a series of oncogenic Ras constructs that we will use. One group is tagged with hemagglutinin (HA), permitting immunoprecipitation and/or detection with anti-HA monoclonal antibody. We have also generated constructs expressing oncogenic Ras with effector site mutations for investigating the precise role of Ras and its downstream effectors. Another series of Ras constructs that we have produced contains the consensus sequence for N-terminal myristoylation, with and without farnesylation-encoding sequences. These constructs will be employed to delineate mechanisms if the results of experiments indicate that Ras may be critical in the effects of Fmev (or lovastatin) on breast cancer cell proliferation.

Discussion

The overall goal of the proposed research is to test the effectiveness of Fmev, an analogue of the naturally-occurring substance mevalonate, in blocking the growth of breast cancer cell lines. We initially established cell culture conditions and methods of quantitative measurement of proliferation and then applied these to studies of the effects of inhibition of mevalonate synthesis (lovastatin experiments) and mevalonate metabolism (Fmev experiments). The data obtained from these studies indicate that breast cancer cell lines share some characterisitics with leukemia and lymphoma cell lines, suggesting that Fmev may prove to be a useful introduction to the development of targeted chemotherapeutic strategies.

Lovastatin, an HMG CoA reductase inhibitor, induced apoptosis and suppressed the proliferation of breast cancer cell lines cultured in the presence of serum lipoproteins as an exogenous source of cholesterol. Mevalonate, the immediate product of HMG CoA reductase activity (Figure 1), reversed the inhibitory effects and thereby demonstrated specificity. These studies indicated that breast cancer cells require a non-sterol derivative of mevalonate or its products for normal growth. This characteristic is shared by all other cell lines.[1, 6, 7] The concentration of lovastatin required to prevent proliferation totally (100 μM) was similar in different breast cancer cell lines and higher than that required for many other cells. One possible explanation for this observation is that lower concentrations of lovastatin were insufficient because the cells have an extremely high level of HMG CoA reductase activity. Lovastatin is a competitive inhibitor and thus the intrinsic level of HMG CoA reductase activity will determine the effect of any given intra-cellular concentration of lovastatin. The measured levels of HMG CoA reductase activity are similar

to those observed in cells with a greater sensitivity to inhibition by lovastatin. Alternatively, the findings may reflect a decrease in the capacity of lovastatin to enter the cells or a lesser dependence on the unidentified non-sterol product to maintain cell growth.

Whereas mevalonate effectively restored growth of lovastatin-blocked cells, neither farnesol nor geranylgeraniol, prenyl alcohols that can be used as precursors for the genertion of farnesyl and geranylgeranyl pyrophosphate respectively, was efficacious. In brain cancer cell lines, geranylgeraniol can restore proliferation inhibited by lovastatin.[13] We have shown that when geranylgeraniol is added to lovastatin-blocked cells, proteins that are normally geranylgeranylated are membrane-associated indicating the capacity of geranylgeraniol to provide precursors for the process of prenylation (geranylgeranylation). Breast cancer cells are similar to other leukemia and lymphoma cell lines in the failure of geranylgeraniol to restore proliferation to lovastatin-blocked cells (unpublished data). The results obtained in breast cancer cell lines thus reinforce our previous data and our hypothesis that the essential non-sterol mevalonate product is derived directly from mevalonate or its phosphates. These combined results from studies in leukemia and lymphoma cells and in breast cancer cells are being prepared for publication.

Fmev inhibited the proliferation of breast cancer cell lines in a concentration-dependent manner. Unlike lovastatin, however, there was variability in not only the effective concentration but also in the mechanism whereby inhibition was produced. Thus, in MCF-7 cells, inhibition of proliferation was profound when 500 μM Fmev was added to cultures. Lower concentrations of Fmev demonstrated enhanced inhibitory efficacy when exogenous mevalonate was added. In addition, suppression of the endogenous synthesis of mevalonate with lovastatin, restored MCF-7 cell growth. Taken together, these results indicate that the effect of Fmev on MCF-7 is mediated by the accumulation of an inhibitor derived from mevalonate or the mevalonate phosphates. In MDA-468-T cells, however, a more complex pattern emerged. Higher concentrations of Fmev were required for comparable levels of inhibition and there was only partial reversal with lovastatin. These observations suggest the possibility of two pathways of inhibition in MDA-468-T cells, one of which is associated with the accumulation of a mevalonate or mevalonate phosphate derivative.

Two other experimental systems provided results that were consistent with the hypothesis of greater accumulation of a mevalonate inhibitory substance in MCF-7 cells. HMG CoA reductase activity in the relatively sensitive MCF-7 cells was more than 3-fold higher than in the relatively resistant MDA-468-T cells. With higher intrinsic levels of HMG CoA reductase, mevalonate synthesis would be higher in MCF-7 cells. Consequently, the accumulation of an inhibitor would occur to a greater extent for any given rate of inhibition of mevalonate metabolism by Fmev. The results obtained by measurements of mevalonate metabolism also indirectly support the disparity in HMG CoA reductase activity between the different cell lines. Thus, comparably more labeled mevalonate is incorporated into protein when the total intra-cellular mevalonate pool is diminished. Althought the total mevalonate incorporation into MCF-7 and MDA-468-T cells was similar there were fractional differences with greater percentage incorporation into protein in MDA-468-T cells. This observation therefore suggests that MDA-468-T cells have a smaller intra-cellular pool of mevalonate when compared with MCF-7 cells and therefore a lower accumulation rate of mevalonate metabolites when the cells are blocked with Fmev.

With the development of a knowledge base that provides information regarding mevalonate synthesis and metabolism in breast cancer cell lines, we have now turned our attention to the interaction of Ras with these compounds. Our initial studies have demonstrated that endogenous levels of Ras can be detected in the breast cancer cell lines that we have studied, albeit in low abundance. Furthermore, Ras was appropriately membrane-associated in normal cells but remained in the cytosol in lovastatin-blocked cells because of inhibition of post-translational processing (farnesylation). We are now focussing on developing a highly efficient immunoprecipitation procedure. To do so, we have turned to cells transfected with tagged Ras. Once the procedure is optimized, our plan is to study Ras synthesis and degradation in Fmev- and lovastatin-blocked breast cancer cell lines. If we are unable to draw solid conclusions from studies of endogenous Ras, we will rely on data obtained from transfection experiments. Breast cancer cell lines will be transfected with various Ras constructs designed to associate with the membrane in a normal manner or by N-terminal fatty acylation (myristoylation). By comparing the data obtained in normal (non-transfected) cells and those expressing the specially-designed constructs, we anticipate that we will determine the relationship between Ras turnover and inhibition of proliferation. If the synthesis of Ras is perturbed, then transcriptional and translational aspects will be examined. If the degradation of Ras is affected, then the processes that control proteosomal destruction of proteins will be analyzed.

***Recommendations** in relation to Statement of Work*

The initial phases of the proposed work have been accomplished. Thus, we have successfully cultured breast cancer cell lines, set up methods to quantitate proliferation and determined the effects of lovastatin and Fmev. Data demonstrating that lovastatin induces apoptosis in breast cancer cell lines and that neither farnesol nor geranylgeraniol can reverse the effects of lovastatin are near completion and being prepared for publication. Fmev is not commercially available and our limited supplies are being conserved. All experimental protocols are being carried to completion with lovastatin inhibition before any Fmev studies are undertaken. By using this approach, the expenditure on Fmev synthesis is being minimized. Nevertheless, we anticipate that the results of studies on the effects of Fmev and lovastatin on Ras synthesis and degradation will be forthcoming shortly. This will permit the commencement of studies proposed for the final two years of the current proposal.

CONCLUSIONS of Progress Report

The proliferation of breast cancer cell lines *in vitro* is suppressed by lovastatin, an inhibitor of HMG CoA reductase, and by Fmev, an inhibitor of phosphomevalonate decarboxylase. Lovastatin-mediated inhibition induces apoptosis of the breast cancer cells. The inhibitory process is specific since provision of mevalonate, the product of HMG CoA reductase activity, totally restores cell growth. The mechanism of lovastatin inhibition is one of preventing the formation of a necessary mevalonate-derived compound. Products of mevalonate including cholesterol and the prenylation precursors farnesol and geranylgeraniol are ineffective at restoring proliferation and thus do not represent the essential non-sterol mevalonate product. Fmev, in contrast, suppresses the proliferation of MCF-7 breast cancer cells by causing the accumulation of a mevalonate-derived inhibitor. Thus, lovastatin, by blocking mevalonate synthesis and decreasing the accumulation of the inhibitor, restores cell growth. These results indicate that therapies which are based on these studies

may permit more effective inhibition of the growth of breast cancer cells *in vivo*. The future development of more specific targeted therapies may be enhanced by the studies planned for the next two years when the mechanism(s) of inhibition will be investigated and analyzed.

References

1. Cuthbert JA and PE Lipsky: Negative regulation of cell proliferation by mevalonate or one of the mevalonate phosphates. *J. Biol. Chem.* **266:** 17966-17971, 1991.
2. Cuthbert JA and PE Lipsky: Suppression of the proliferation of Ras-transformed cells by fluoromevalonate, an inhibitor of mevalonate metabolism. *Cancer Res.* **55:** 1732-1740, 1995.
3. Cuthbert JA and PE Lipsky: Regulation of proliferation and Ras localization in transformed cells by products of mevalonate metabolism. *Cancer Res* **57:** 3498-3505, 1997.
4. Quistad GB, DC Cerf, DA Schooley and GB Staal: Fluoromevalonate - an inhibitor of insect juvenile hormone biosynthesis. *Pr Nauk Inst Chem Org Fiz Politech Wroclaw* **22:** 163-168, 1981.
5. Goldstein JL and MS Brown: Regulation of the mevalonate pathway. *Nature* **343:** 425-430, 1990.
6. Kaneko I, Y Hazama-Shimada and A Endo: Inhibitory effects on lipid metabolism in cultured cells of ML-236B, a potent inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme-A reductase. *Eur. J. Biochem.* **87:** 313-321, 1978.
7. Cuthbert JA and PE Lipsky: Inhibition by 6-fluoromevalonate demonstrates that mevalonate or one of the mevalonate phosphates is necessary for lymphocyte proliferation. *J. Biol. Chem.* **265:** 18568-18575, 1990.
8. Cuthbert JA and PE Lipsky: Provision of cholesterol to lymphocytes by high density and low density lipoproteins. Requirement for low density lipoprotein receptors. *J. Biol. Chem.* **262:** 7808-7818, 1987.
9. Nave JF, H d'Orchymont, JB Ducep, F Piriou and MJ Jung: Mechanism of the inhibition of cholesterol biosynthesis by 6- fluoromevalonate. *Biochem. J.* **227:** 247-254, 1985.
10. Reardon JE and RH Abeles: Inhibition of cholesterol biosynthesis by fluorinated mevalonate analogues. *Biochemistry* **26:** 4717-4722, 1987.
11. Barbacid M: *ras* genes. *Annu. Rev. Biochem.* **56:** 779-827, 1987.
12. Bos J. L.: The *ras* gene family and human carcinogenesis. *Mut. Res.* **195:** 255-271, 1988.
13. Crick DC, DA Andres, R Danesi, M Macchia and CJ Waechter: Geranylgeraniol overcomes the block of cell proliferation by lovastatin in C6 glioma cells. *J Neurochem* **70:** 2397-2405, 1998.

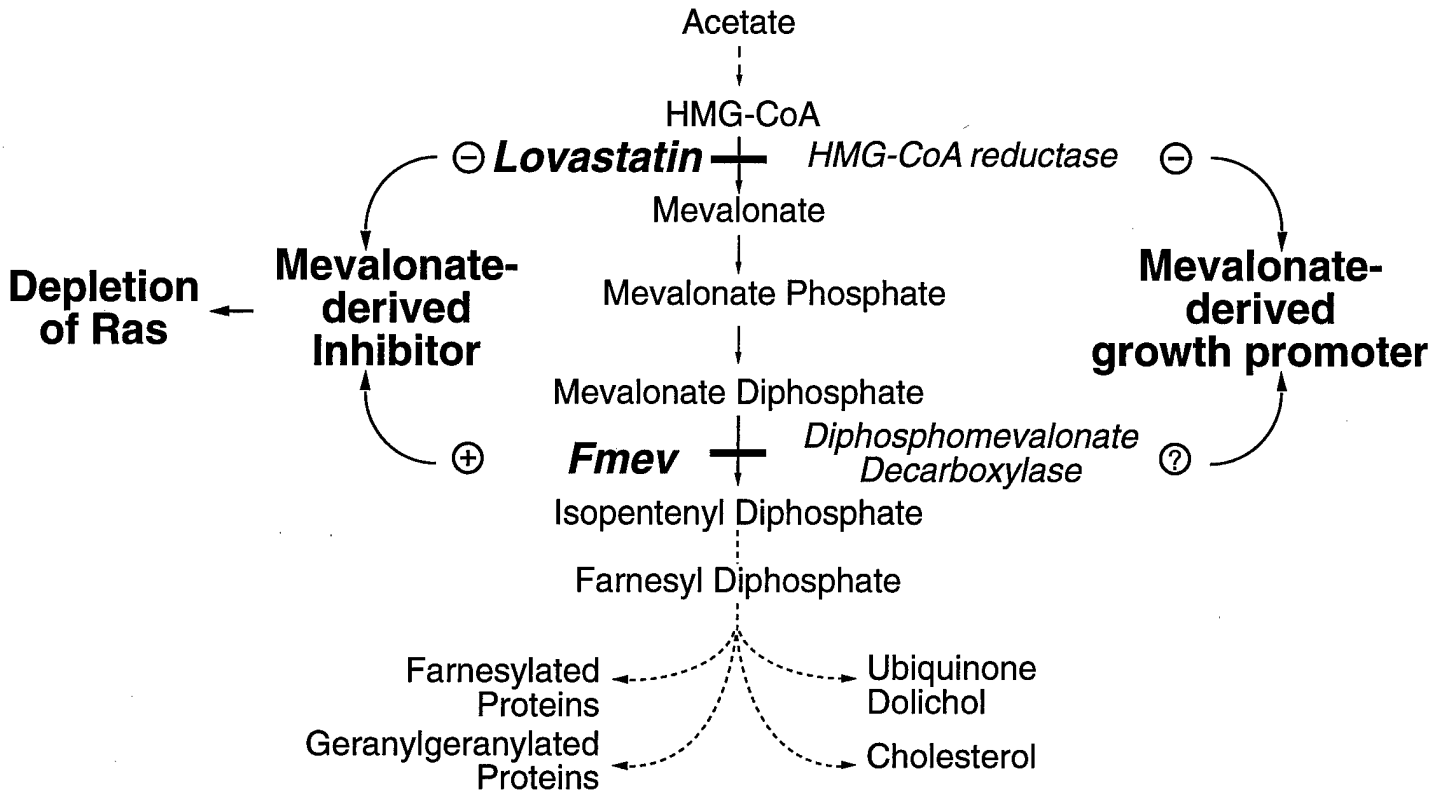

Figure 1: Mevalonate synthetic and metabolic pathways

[CANCER RESEARCH 57, 3498–3505, August 15, 1997]

Regulation of Proliferation and Ras Localization in Transformed Cells by Products of Mevalonate Metabolism[1]

Jennifer A. Cuthbert[2] and Peter E. Lipsky

Department of Internal Medicine, The University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235-9151

ABSTRACT

Lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl (HMG) CoA reductase, and 6-fluoromevalonate (Fmev), an inhibitor of diphosphomevalonate decarboxylase, blocked the synthesis of downstream mevalonate products, including prenyl-derived lipids, and prevented membrane localization of Ras in the myeloid cell line U-937. In contrast to lovastatin, which induced cytosol localization of Ras in U-937 cells, Fmev failed to increase cytosolic Ras and also completely prevented the proliferation of U-937 cells. Growth of U-937 cells was restored by the addition of lovastatin to Fmev-blocked cells. These results implied that a product of mevalonate metabolism proximal to isopentenyl diphosphate was responsible for the suppression of proliferation. To delineate the action of this endogenous inhibitor of cell proliferation and determine the relationship between its impact on Ras localization and cell proliferation, the effect of Fmev on a variety of leukemia- and lymphoma-derived cells was examined. Whereas Fmev blocked the growth of these cell lines, there were more than 50-fold differences in the concentrations required to inhibit the growth of individual cell lines by 90%. Regardless of its effect on cell proliferation, the biochemical effect of Fmev was similar. Thus, Fmev uniformly prevented the conversion of radiolabeled mevalonate to isopentenyl diphosphate and other downstream products, including synthesis of sterol and nonsterol lipids and prenylation of proteins. A correlation was noted between higher intrinsic rates of mevalonate synthesis by a cell and susceptibility to inhibition by Fmev. Thus, sensitivity of a cell line to inhibition by Fmev was associated with markedly increased rates of HMG CoA reductase activity that were further increased by incubation with Fmev. Whereas Fmev depleted cellular levels of the prenylated protein Ras in the sensitive cell line U-937, there was no depletion of cellular Ras levels in the resistant cell line EL-4, but rather, there was a shift of Ras from membrane to cytosol, as expected for inhibition of prenylation. These results suggest that leukemic cells with increased HMG CoA reductase activity produce increased levels of an endogenous mevalonate-derived inhibitor that leads to Ras depletion and suppression of cell growth. As a result, inhibition of the growth of these transformed cells might be specifically accomplished by Fmev.

INTRODUCTION

Ras, a M_r 21,000 guanine nucleotide-binding protein, is activated by signal transduction pathways involved in growth and differentiation (1). Mutations that constitutively activate Ras are found in ~20% of all human tumors (1, 2) and are particularly common in pancreatic (3) and colon (4) cancer. Cellular transformation by mutationally activated Ras requires plasma membrane association of the oncogenic protein (5–7). This observation has been exploited in the development of potential cancer chemotherapeutic agents that block membrane association of Ras (8–11).

Initially, newly synthesized Ras is localized in the cytosol (12–14). A series of posttranslational modifications of the COOH terminus results in plasma membrane targeting (12–14). These include the addition of a 15-carbon farnesyl lipid to the cysteine at amino acid position 186, the removal of the three COOH-terminal amino acids at positions 187–189, and carboxymethylation of the new COOH-terminal cysteine. In addition, either palmitylation of other cysteine residues in the COOH terminus (H-Ras, N-Ras, and K-RasA) or a polybasic domain (K-RasB) is important in enhancing membrane association (7). These processes occur stepwise, and the first step, that of farnesylation of the full-length polypeptide, is thereby essential for plasma membrane localization (12–14). Thus, compounds and mutations that block the process of farnesylation interfere with the transformation and proliferation that are dependent upon mutationally activated Ras.

Lovastatin, a specific inhibitor of HMG[3] CoA reductase, blocks farnesylation of Ras (12, 13) because HMG CoA reductase catalyzes the synthesis of mevalonate, the precursor of the farnesyl lipid moiety (Ref. 15; see Fig. 7 for biochemical pathways). Unfarnesylated Ras remains in the cytosol of lovastatin-blocked cells. In addition, inhibitors of the enzyme farnesyltransferase prevent posttranslational modification and membrane association of Ras (8, 9). Farnesyltransferase inhibitors prevent cell growth that is dependent on mutationally activated Ras (8–11). We have demonstrated that Fmev, a fluorinated mevalonate analogue that blocks the conversion of mevalonate diphosphate to isopentenyl diphosphate (16, 17), also inhibits farnesylation of Ras (18, 19). Like lovastatin, Fmev prevents membrane localization of Ras (19). Unlike lovastatin, however, Fmev does not induce the accumulation of Ras in the cytosol (19). Moreover, Fmev also induces the activity of an endogenous inhibitor of cellular proliferation (19, 20). This inhibitor is a product of mevalonate proximal to isopentenyl diphosphate (19, 20). The current studies were undertaken to examine the potential dysregulation of this inhibitor of cellular growth in various transformed cell lines and determine the relationship of its impact on Ras localization and cellular growth.

MATERIALS AND METHODS

Cell Culture and Measurement of DNA, Sterol, and Mevalonate Synthesis. The following leukemia- and lymphoma-derived cell lines were obtained from the American Type Culture Collection: the B-cell line Daudi (Burkitt lymphoma ATCC CCL 213); the lymphoma cell line EL-4 (ATCC TIB 39); the acute monocytic leukemia cell line THP-1 (ATCC TIB 202); the acute promyelocytic leukemia line HL-60 (ATCC CCL 240); and the histiocytic lymphoma-derived myeloid cell line, U-937 (ATCC CRL 1593). The normal human B-lymphoblastoid cell line, MarB, that had been generated by transformation of peripheral blood mononuclear cells with Epstein-Barr virus using standard techniques (21) was obtained from Dr. S. Zwillich (University of Texas Southwestern Medical School). For some experiments, the Epstein-Barr virus-transformed human B-lymphoblastoid cell line 526.11 (22) was used. The Jurkat T-cell line was obtained from Dr. L. Davis (University of Texas Southwestern Medical School). All cells were maintained in RPMI-1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% (v/v) iron-supplemented BCS (HyClone Laboratories, Inc., Logan, UT). For some experiments, cells were adapted to medium supplemented with 10% (v/v) LPP, prepared as detailed (18). Fmev was synthesized by CPM Laboratory (Carrollton, TX) using the procedure of Quistadt *et al.* (23). Lovastatin (Merck,

Received 11/18/96; accepted 6/17/97.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Grant CN-108 from the American Cancer Society.

[2] To whom requests for reprints should be addressed. Phone: (214) 648-2666; Fax: (214) 648-5678; E-mail: cuthbert@utsw.swmed.edu.

[3] The abbreviations used are: HMG, 3-hydroxy-3-methylglutaryl; Fmev, 6-fluoromevalonate; BCS, bovine calf serum; LPP, lipoprotein-poor plasma.

[CANCER RESEARCH 55, 1732–1740, April 15, 1995]

Suppression of the Proliferation of Ras-transformed Cells by Fluoromevalonate, an Inhibitor of Mevalonate Metabolism[1]

Jennifer A. Cuthbert[2] and Peter E. Lipsky

Department of Internal Medicine, The University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235-8887

ABSTRACT

Mevalonate is the precursor of a number of different products potentially required for the growth of cells, including the prenylated oncoprotein Ras. To determine whether inhibition of mevalonate metabolism would selectively block proliferation of Ras-transformed cells, 6-fluoromevalonate (Fmev), an inhibitor of diphosphomevalonate decarboxylase, was used to block the synthesis of prenyl-derived lipids and prenylated proteins in interleukin-3 (IL-3)-dependent FDC-P1 cells (control FDC-P1 cells) and FDC-P1 cells transformed with oncogenic Ras (RasDC cells) that proliferated in the absence of IL-3. Fmev completely inhibited synthesis of prenyl-derived lipids and prenylated proteins and blocked proliferation of FDC-P1 and RasDC cells. Restoration of the proliferation of Fmev-blocked FDC-P1 cells required both an exogenous source of cholesterol and prevention of the accumulation of mevalonate and the mevalonate phosphates with lovastatin. In contrast, ongoing IL-3-independent proliferation of Fmev-blocked RasDC cells was not completely restored by providing exogenous cholesterol and preventing the accumulation of inhibitory mevalonate product(s). However, these cells proliferated when cultures were supplemented with IL-3 together with exogenous cholesterol and lovastatin, implying that Fmev had prevented Ras-dependent, IL-3-independent growth. Fmev markedly diminished total cellular Ras in RasDC cells. In contrast, lovastatin depleted membrane-associated Ras and increased cytosolic Ras but did not diminish total cellular Ras. These data indicate that Fmev depletes total cellular Ras and specifically inhibits the autonomous growth of Ras-transformed cells.

INTRODUCTION

In normal cells, Ras, a plasma membrane-associated 21 kDa guanine nucleotide-binding protein, is involved in signal transduction associated with growth and differentiation (1). Interference with the normal function of Ras in cultured cells suppresses cell growth (2–4), whereas introduction of activated Ras can lead to transformation (1). Mutations that constitutively activate a cellular Ras oncoprotein have been found in ~20% of all human tumors (1, 5) and are particularly prevalent in adenocarcinomas of the colon (6) and pancreas (7). Disruption of an activated *Ras* gene in human colon cancer cell lines slows proliferation *in vitro* and suppresses anchorage-independent growth (8), suggesting that therapeutic approaches targeting Ras may be beneficial.

Membrane localization of Ras, which is important for *in vitro* transforming activity, is normally dependent on posttranslational modification of the carboxy terminus by sequential farnesylation, proteolysis, and carboxymethylation (9–19). Since blocking posttranslational farnesylation of Ras precludes subsequent membrane localization (9, 11, 12, 14, 16), it could prevent the transforming potential of oncogenic Ras. Inhibition of the synthesis of farnesyl 1-diphosphate, by blocking the formation of the precursor mevalonate with lovastatin, effectively prevents posttranslational processing of Ras and subsequent membrane localization (11, 12). In addition, selective inhibition of the growth of Ras-transformed cells by inhibitors of farnesyltransferase, the enzyme that modifies Ras by the addition of the farnesyl group, has been reported (20, 21). However, the efficacy of these compounds at inhibiting the growth of malignant cells, where multiple genetic alterations often contribute to the cancer phenotype, is less certain. In contrast, Fmev,[3] a potent inhibitor of the activity of diphosphomevalonate decarboxylase (22, 23), completely blocks protein prenylation (24–26) and inhibits the proliferation of various transformed cells (27) but does not block proliferation of normal cells if cholesterol is provided (26). In these transformed cells, suppression of proliferation is caused by the accumulation of mevalonate or mevalonate phosphate(s) rather than by the inhibition of cholesterol biosynthesis (27). The current studies were carried out to determine whether Fmev would inhibit the growth of Ras-transformed cells by these mechanisms as well as by suppressing the prenylation and membrane association of the mutant cellular oncoprotein.

MATERIALS AND METHODS

Cell Culture and Measurement of DNA, Sterol, and Mevalonate Synthesis and Cell Growth. FDC-P1 (Paterson Laboratories) cells and the ras-transformed FDC-P1 cell line FI NR G4 (28) that has been transfected with a mutant human H-*ras* genomic clone derived from the T24 bladder carcinoma (Ref. 29; RasDC) were generously provided by Dr. H. Scott Boswell (Indiana University School of Medicine, Indianapolis, IN). Proliferation of FDC-P1 cells is dependent on the exogenous growth factor IL-3 (30). However, when transformed with oncogenic Ras, cell growth becomes IL-3 independent (28), thus permitting direct evaluation of the effects of experimental manipulations on Ras-dependent proliferation. All cells were maintained in complete medium and RPMI 1640 (Whittaker Bioproducts Inc., Walkersville, MD) supplemented with 10% v/v iron-supplemented BCS (Sigma Chemical Co., St. Louis, MO). In some experiments, medium was supplemented with 10% v/v LPP, prepared as detailed (26). Concentrated culture supernatant from WEHI-3 cells (final concentration, 2–5% v/v) was used as the source of IL-3 for maintaining FDC-P1 cells. Recombinant IL-3 (DNAX Research Institute, Inc., Palo Alto, CA) was used for some experiments, as indicated. Fmev was synthesized by CPM Laboratory (Carrollton, TX) using the procedure of Quistad *et al.* (31). Lovastatin (Merck, Sharp and Dohme, Rahway, NJ) and the sodium salt of mevalonate were prepared as reported previously (32) and added where indicated. Preliminary experiments demonstrated that lovastatin inhibited endogenous sterol synthesis, measured by the incorporation of radiolabeled acetate into digitonin-precipitable sterols (32), in a concentration-dependent manner (control, 274.4 ± 12.0 pmol/h/10^6 cells [mean ± SEM, n = 3], 0.5 μM lovastatin, 56.3 ± 0.4 pmol/h/10^6 cells [80% inhibition]; 5 μM lovastatin, 12.1 ± 1.1 pmol/h/10^6 cells [96% inhibition]; 50 μM lovastatin, 1.8 ± 0.3 pmol/h/10^6 cells [99% inhibition]).

For measurements of DNA synthesis, cells were cultured (≤5 × 10^3 cells/well) in triplicate in microtiter plates with additions as indicated in the individual experiments; in preliminary experiments, this concentration of cells allowed maximum proliferation during the ensuing 1–4-day incubation. DNA synthesis was measured by the incorporation of [^{3}H]thymidine as described (32), after the addition of 5-fluorodeoxyuridine (10 μM) to block endogenous thymidine synthesis. In some experiments, cells were cultured at a lower

Received 12/27/94; accepted 2/13/95.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Grant CN-108 from the American Cancer Society and Public Health Service Grant AI-17653 from the NIH.

[2] To whom requests for reprints should be addressed, at Liver Unit, Department of Internal Medicine, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-8887.

[3] The abbreviations used are: Fmev, 6-fluoromevalonate; FDC-P1 cells, factor-dependent continuous cell line; RasDC cells, oncogenic Ras-dependent FDC-P1 cells; BCS; bovine calf serum; LPP, lipoprotein-poor plasma; IL-3, interleukin-3; HMG CoA, 3-hydroxy-3-methylglutaryl-CoA; PAGE, polyacrylamide gel electrophoresis.

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 266, No. 27, Issue of September 25, pp. 17966-17971, 1991
Printed in U.S.A.

Negative Regulation of Cell Proliferation by Mevalonate or One of the Mevalonate Phosphates*

(Received for publication, February 13, 1991)

Jennifer A. Cuthbert‡ and Peter E. Lipsky

From the Department of Internal Medicine, The University of Texas Southwestern Medical Center, Dallas, Texas 75235-8887

The role of mevalonate and its products in the regulation of cellular proliferation was examined using 6-fluoromevalonate (Fmev), a compound that blocks the conversion of mevalonate pyrophosphate to isopentenyl pyrophosphate. Fmev suppressed DNA synthesis by a variety of transformed and malignant T cell, B cell, and myeloid cell lines. In contrast to results previously reported with mitogen-stimulated human peripheral blood T cell DNA synthesis, low concentrations of low density lipoprotein (LDL) alone could not restore proliferation to these cell lines. The same concentrations of LDL were able to provide sufficient cholesterol and support the growth of all cell lines when mevalonate synthesis was blocked with a specific inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, lovastatin. Fmev-mediated inhibition was totally prevented in some but not all cell lines when the concentration of exogenous LDL was increased 5–10-fold above that required to permit proliferation of lovastatin-blocked cells. Residual HMG-CoA reductase activity of cells cultured with LDL inversely correlated with the restoration of growth to Fmev-blocked cultures. Confirmation of the critical role of HMG-CoA reductase activity and mevalonate synthesis in the inhibition of cellular proliferation by Fmev was obtained by demonstrating that the specific inhibitor of this enzyme, lovastatin, restored proliferation of Fmev-blocked cells. Furthermore, supplementation of cultures with mevalonate, the product of HMG-CoA reductase activity, markedly inhibited proliferation of Fmev-blocked cells. These findings indicate that mevalonate or one of the mevalonate phosphates, which accumulates in Fmev-blocked cells, is a critical negative regulator of cellular proliferation.

Proliferation of all cells is known to require at least two products synthesized from mevalonate, only one of which, cholesterol, has been identified (reviewed in Ref. 1). Other mevalonate-derived products that are potential candidates for the non-sterol compound include tRNA containing adenine modified by the addition of an isopentene group originating from mevalonate, and the lipids, ubiquinone and dolichol (1). In addition, recent studies have identified a number of proteins with mevalonate-derived prenyl groups attached post-translationally (2–6). However, none of these prenylated proteins or other compounds has been demonstrated to be the non-sterol product derived from mevalonate that is essential for cell proliferation.

In previous studies, the most commonly used experimental approach to define the role of mevalonate in cell proliferation has been to examine the effect of suppression of mevalonate synthesis (7–10). This has been achieved by blocking the activity of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA)[1] reductase with highly potent, specific inhibitors such as compactin (7–9) or lovastatin (10). Addition of large concentrations of these inhibitors blocks cell proliferation, even when sufficient exogenously supplied cholesterol is present for membrane synthesis (7–10). Cell growth is restored by supplementation of the culture medium with trace quantities of mevalonate, in addition to cholesterol, indicating that a non-sterol product of mevalonate is necessary for cell growth (1, 10).

We have taken an alternative approach to analyze the non-sterol product of mevalonate necessary for cell growth by using the mevalonate analogue, 6-fluoromevalonate (Fmev), which prevents the decarboxylation of mevalonate pyrophosphate to isopentenyl pyrophosphate. Fmev does not suppress synthesis of mevalonate and the mevalonate phosphates, although synthesis of other more distal products, including prenylated proteins and the lipids cholesterol, ubiquinone and dolichol, is inhibited (10). Unlike suppression of cell growth by lovastatin and compactin, Fmev-mediated inhibition of mitogen-stimulated human T cell DNA synthesis was completely overcome by low density lipoprotein (LDL) alone. This finding indicated that mevalonate or one of the mevalonate phosphates was the source of the factor necessary for growth of T cells.

Earlier studies have suggested that a product of mevalonate might also negatively influence cellular function or growth (11, 12). Thus, mevalonate was cytotoxic for a Chinese hamster ovary cell variant that manifested specifically increased mevalonate transport (11). Similarly, mevalonate inhibited the growth of a human adrenocortical carcinoma cell line stimulated by inhibition of HMG-CoA reductase with lovastatin (12). Neither the nature of the inhibition nor the inhibitory mevalonate-derived moiety was identified in these studies. The capacity of Fmev to cause the accumulation of mevalonate and its immediate phosphate derivatives (13, 14), but not limit the availability of the non-sterol product of mevalonate necessary for cell growth (10), made it possible to examine the negative regulatory action of mevalonate. The results indicate that accumulation of mevalonate from either

* This work was supported by National Institutes of Health Grant AI17653 and by the American Heart Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Liver Unit, Dept. of Internal Medicine, UT Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-8887.

[1] The abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; Fmev, 6-fluoromevalonate; LDL, low density lipoprotein.

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 265, No. 30, Issue of October 25, pp. 18568-18575, 1990
Printed in U.S.A.

Inhibition by 6-Fluoromevalonate Demonstrates That Mevalonate or One of the Mevalonate Phosphates Is Necessary for Lymphocyte Proliferation*

(Received for publication, April 16, 1990)

Jennifer A. Cuthbert‡ and Peter E. Lipsky

From the Department of Internal Medicine, The University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235-8887

The sterol synthesis inhibitor 6-fluoromevalonate (Fmev) was used to explore the role of mevalonate products in lymphocyte proliferation. Fmev blocks the synthesis of isopentenyl pyrophosphate and all more distal products in the sterol pathway. When cells were cultured in lipoprotein-deficient medium, Fmev (200 μM) completely inhibited mitogen-stimulated human lymphocyte proliferation, quantified by measuring DNA synthesis. The addition of low density lipoprotein (LDL) restored lymphocyte responses to normal, whereas mevalonate was totally ineffective. Similar results were obtained with concentrations of Fmev up to 1 mM. These results contrast with those observed when sterol biosynthesis was blocked with lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase. When lymphocyte proliferation was blocked with lovastatin (5 μM), either high concentrations of mevalonate or LDL together with low concentrations of mevalonate was required to restore responses. In contrast, neither LDL nor low concentrations of mevalonate when alone was able to restore lymphocyte DNA synthesis in cultures blocked with 5 μM lovastatin. The effect of Fmev on the capacity of exogenous mevalonate to restore proliferation of lovastatin-blocked lymphocytes was directly examined. Fmev had no effect on the capacity of LDL plus low concentrations of mevalonate to restore DNA synthesis to lovastatin-blocked lymphocytes, indicating that the synthesis of the necessary factor from mevalonate was unaltered by Fmev. Fmev profoundly blocked lymphocyte endogenous sterol synthesis, decreasing incorporation of radiolabeled acetate into digitonin-precipitable sterols by up to 98%. LDL did not alter the capacity of Fmev to block sterol synthesis. The possibility that Fmev allowed shunting of endogenous mevalonate into essential lipid products was assessed by examining the incorporation of radiolabeled mevalonate. Fmev (200 μM) inhibited the incorporation of mevalonate into all lipids, including ubiquinone, dolichol, and other non-sterol lipids by up to 98%, and this was not altered by LDL. Furthermore, Fmev (200 μM) suppressed the incorporation of radiolabeled mevalonate into protein by up to 97%. These data confirm that a product of mevalonate is essential for cell proliferation. However, the results indicate that the required product is directly synthesized from mevalonate or mevalonate phosphates rather than from a more distal isoprenoid metabolite.

Proliferation of all cells is known to require at least two products of mevalonate metabolism. Although one of these has been clearly shown to be cholesterol (1–4), the other product has only been defined indirectly and has not been identified (5–9). Thus, when mevalonate synthesis is inhibited by blocking the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)[1] reductase, cellular proliferation is completely prevented (5–7). The addition of exogenous mevalonate is able to restore proliferation (5–7). However, cholesterol alone is ineffective, thereby demonstrating the need for a second product of mevalonate metabolism other than cholesterol (5–7). The identification of the non-sterol product of mevalonate that is necessary for cellular proliferation has proven to be difficult.

Among the mevalonate-derived products that may be important for cellular proliferation are transfer RNA species, isoprenoids, and proteins that are post-translationally modified by mevalonate-derived moieties (10–12). Studies in baby hamster kidney cell lines initially suggested that adenine, containing an isopentenyl group originating from mevalonate, was the factor necessary for DNA synthesis (13). However, the capacity of isopentenyl adenine to rescue proliferation of cells blocked by inhibitors of HMG-CoA reductase has not been confirmed, suggesting that other metabolites of mevalonate might be required (14). Candidate compounds include the lipids, dolichol and ubiquinone, that are synthesized from isoprene groups originating from mevalonate (15–19). Dolichol plays a role in the glycosylation of proteins and ubiquinones are involved in the mitochondrial respiratory chain (20, 21). More recent studies have shown that certain proteins are post-translationally modified by the covalent attachment of mevalonate-derived isoprene groups (22–26). The identification of some of these proteins as nuclear lamin B (22, 23) and guanine nucleotide-binding proteins (24–26) has led to the speculation that one or more of these proteins may be the mevalonate product necessary for cellular proliferation (9, 23–26). None of these compounds, however, has been clearly identified as the mevalonate-derived factor required for cell growth.

* This work was supported by Grant AI17653 from the National Institutes of Health and by a grant from the American Heart Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Liver Unit, Dept. of Internal Medicine, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-8887. Tel.: 214-688-3440.

[1] The abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; Fmev, 6-fluoromevalonate; LDL, low density lipoprotein; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin.